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# *To My Wife*

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The present work comprises a survey of eight papers published previously by the same author, as well as experimental work meant to shed light on current problems in the same field. In order to facilitate a more uniform presentation, the papers listed below will be cited in the text by Roman numbers.

I. BÁNHIDI, Z. G., Some Aspects of the Nutrition of *Lactobacillus fermenti* 36 in the Tube Assay of Thiamine. Acta Chem. Scand. 1958, *12*, 517–527.

II. BÁNHIDI, Z. G., Improvements in the Agar-plate Test for Thiamine Factors with *Lactobacillus fermenti* 36. Analyst 1959, *84*, 657–659.

III. BÁNHIDI, Z. G., Über die Wachstumswirkung von Thiamindisulfid auf Lactobacillus fermenti 36 im Agarplatten-Test Biochem. Z. 1959, *332*, 77–80.

IV. BÁNHIDI, Z. G., Activation of the Disulfide Forms of Thiamine and its Phosphates as Growth Factors for *Lactobacillus fermenti*. J. Bacteriol. 1960, *79*, 181–190.

V. BÁNHIDI, Z. G., Microbiological Activity of Some Mixed Disulfides of Thiamine for *Lactobacillus fermenti*. Acta Chem. Scand. 1959, *13*, 1901–1903.

VI. BÁNHIDI, Z. G., Thiamine Pantetheine Disulfide as a Microbial Growth Factor. Acta Chem. Scand. 1959, *13*, 1904–1905.

VII. BÁNHIDI, Z. G., Growth promotion of some heterofermentative Lactobacilli with different derivatives of thiamine. Arkiv Kemi 1960, *15*, 229–239.

VIII. BÁNHIDI, Z. G., Weight increase in the rat due to thiamine disulfide activation by ascorbic acid. Internat. Z. Vitaminforsch., 1960. *30*, 305–322.

# INTRODUCTION

> "It is discouraging that cocarboxylase, although one of the first coenzymes to be discovered, has generally resisted attempts to unravel its fundamental mechanism of action." (NEILANDS and STUMPF, 1958.)

It took half a century from TAKAKI's first observation (1885) that beri-beri was a dietary deficiency and not a contagious disease to the realization that cocarboxylase is identical with the pyrophosphate of thiamine (LOHMANN and SCHUSTER, 1937), the principle which cures this fatal insufficiency in minute doses. Interest in this factor, which led to the introduction of the "vitamin" concept in physiology, is still increasing as is testified by excellent monographs (WILLIAMS and SPIES, 1938; SEBRELL and HARRIS, 1954; GUNSALUS, 1956; WESTERBRINK, 1958) and recent doctoral theses (HJARDE, 1950; IACONO, 1954; FORSANDER, 1956; KIESSLING, 1957).

Thiamine, like other B vitamins of established function, exerts its activity *in vivo*, mostly if not exclusively after esterification to its pyrophosphate by participating in some vital reactions of the intermediary metabolic pattern of all living cells. Thus, it is essential in anoxydative and oxydative decarboxylation of $\alpha$-keto acids, in the transketolase reactions of the hexose-monophosphate shunt, and in acetoine formation. More than twenty reaction types were presented in the compilation of SEBRELL and HARRIS (1954). All these transformations postulate an intermediary active aldehyde form in which an acyl carbanion (R–CO–) is attached to the thiamine (more correctly to the cocarboxylase) molecule at a site not definitely identified despite several theories and model experiments. Only the recent modified theory of BRESLOW (1958) promises a settlement of this question. It should be recognized, however, that some of the earlier theories on thiamine function have at least as much experimental support as that of BRESLOW and that even after final proof of the rightness of the BRESLOW model, the existence of more than one function of the thiamine molecule might be postulated.

Our aim was to investigate the action of different thiamine derivatives (mainly disulfides) on microbial cells in order to contribute to a more specific definition of thiamine function in these cells, as well

as, after extension of the experience gained, in higher organisms. The present work comprises a survey of results reported in eight earlier papers (listed on the inside front cover) and experimental data completing these studies.

## MICROBIOLOGICAL ASSAY OF THIAMINE AND ITS DERIVATIVES

Those working with thiamine are usually bewildered by the difficulties encountered when analyzing this relatively simple and easily synthetizable compound. Because of the interference of alien substances and because of the existence of several inactive forms of thiamine, most colorimetric and fluorometric methods are unreliable. The conventional thiochrome technique of thiamine estimation is not devoid of these inborn analytical errors as shown by comparing the quantitative results obtained by different laboratories with the same natural product (SEBRELL and HARRIS, 1954).

It is a historical coincidence that thiamine was not only the first vitamin discovered, but also the first assayed microbiologically with the aid of *Phycomyces blakesleeanus*. This organism was chosen by SCHOEPFER (1935) among many species of lower fungi which are exacting in respect of thiamine nutrition. While time-consuming as an analytical method, this strain was extensively used for the establishment of the nutritional specificity of the thiamine molecule, besides bacterial growth tests and yeast fermentation methods critically reviewed by SNELL (1950) in his basic work on microbial vitamin assays. Recently *Kloeckera brevis* (HOFF-JØRGENSEN and HANSEN, 1955) and *Lactobacillus viridiscens* (DEIBEL, EVANS and NIVEN, 1957) were recommended for analytical purposes because of disturbing intermittent variations in the otherwise more generally accepted test of SARETT and CHELDELIN (1944) based on *Lactobacillus fermenti* (strain 36) as test organism. Under these auspices our work started with the reinvestigation of the SARETT and CHELDELIN method in the search for a reliable analytical tool. It was found that tryptophane is an essential nutrient for *L. fermenti* and that the alkali-treated peptone supplement of the SARETT and CHELDELIN medium was dispensable if this amino acid was added to the basal medium in sufficient amounts to complete the acid-hydrolyzed casein com-

ponent, the tryptophane ingredient of which was destroyed during hydrolysis (I). Leaving out the peptone supplement, a chemically defined basal medium could be prepared which helped later on in the detection of minute nutritional differences. Since the composition of our basal medium was slightly changed in the course of our continued work, we refer to the formula in its present form (IV, VII, VIII). It should be emphasized that all other technical details of the original paper (I) must be carefully considered for a successful test.

At the time of our work on the improvement of the tube test for thiamine with *L. fermenti*, we could not explain the actual effect of cysteine or other reducing substances on the utilization of thiamine and we simply registered the beneficial effect of these agents on the assay procedure like other workers (FANG and BUTTS, 1953; RAMSEY and LANKFORD, 1956; TERROINE, 1957). We had cystine in our first basal medium according to SARETT and CHELDELIN in the belief that one more nutrient might be favorable even if not essential. The paper of KOSER and THOMAS (1955) was not considered at the time. These authors long ago dealt with the question of the dispensability of cysteine for many Lactobacilli, amongst others, *L. fermenti* ATCC 9338, the strain also used by us.

The seemingly unimportant question of the presence of cystine (or cysteine) in the basal medium soon came, however, into the focus of our attention, since this was found to be a key component for the utilization of the disulfide forms of thiamine (III, IV).

For a successful agar plate test the addition of Tween 80 to the basal medium was found to be necessary (II). Growth zones were hardly visible without this component, which is otherwise dispensable in the tube test, where it exerts only a slight effect on thiamine utilization and a more marked one on the utilization of the thiazole part of the molecule according to MACIASR (1958). Under the prescribed conditions (II) the agar plate test for thiamine with *L. fermenti* is one of the easiest to perform and a most readable agar test. The need for such a method was often emphasized (e.g. ANALYTICAL METHODS COMMITTEE, 1954). There were actually reports on successful agar plate tests with *L. fermenti* (IACONO, 1954; MARTEN, 1955). However, the technical details given must have been insufficient, as we could not reproduce their results.

The agar plate test (II) was extensively used by us for bioautographic detection of different thiamine derivatives according to the

principles introduced by WINSTEN and EIGEN (1948) and our application of these principles to the folic acid group of factors (BÁNHIDI and ERICSON, 1953). Paper chromatograms were usually made in duplicate and one was developed bioautographically on a *L. fermenti* plate with basal medium containing cysteine and the other in a similar manner with medium depleted of cysteine. In this way differentiation could be made between spots containing factors active *per se* for the bacterial cells and those requiring cysteine activation prior to utilization. The term "selective bioautography" was introduced for this technique (VII) in contrast to "comparative bioautography" applying to plates seeded with different microorganisms sensitive to different members of the same group of factors.

## THE BIOLOGICAL SPECIFICITY OF THE THIAMINE MOLECULE

Among the B vitamins thiamine was considered by many workers to be a rather exceptional entity because of its "exceptional specificity" in biological systems (SCHMELKES, 1939). Large numbers of different thiamine analogues were tested during the last few decades for their growth-promoting value in microorganisms and experimental animals. For the latter the curative test was often used. Also inhibitory analogues of thiamine were synthesized and tested with the hope of obtaining information about the mode of action of the thiamine molecule. An exhaustive survey of these efforts was presented by BARTON and ROGERS (1950), who came to the conclusion that "for many microorganisms the structural specificity of vitamin $B_1$" (thiamine) "is substantially the same as for animals".

The test of thiamine analogues on microorganisms implies, naturally, rather well-defined experimental conditions compared with those in animal tests, especially nutritional trials. Working with *Phycomyces blakesleeanus* ROBBINS and KAVANAGH (1938*a* and 1938*b*) concluded that in the thiamine molecule (Fig. 1*a*) the 6′-amino and 5′-monosubstituted methyl groups of the pyrimidine moiety are important, while in the thiazole portion the 2-H must be unaltered and the hydroxyl of the 5-$\beta$-hydroxyethyl group is also important.

BONNER and ERICKSON (1938) came to a similar conclusion emphasizing the requirement of free nitrogen in the thiazole part, able

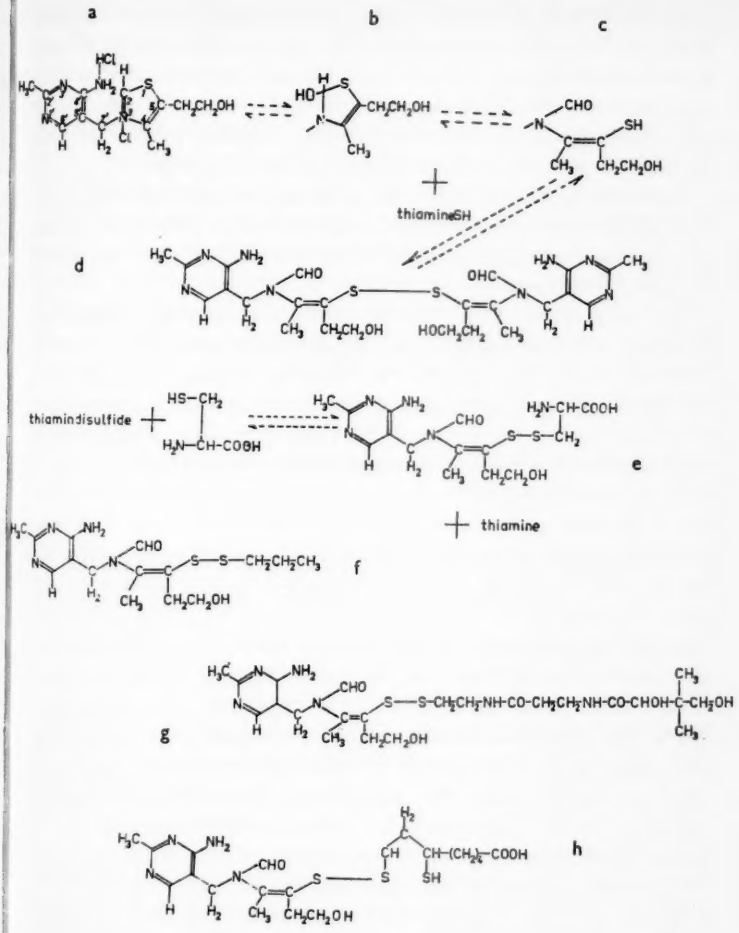


FIG. 1.

to form a quaternary salt with the pyrimidine portion. All authors agree that both the pyrimidine and the thiazole are important for *Phycomyces*, which can otherwise easily synthesize thiamine by coupling the two moieties.

Against the high specificity of thiamine only SCHULTZ (1940, 1941) raised a contrary opinion, stating that several analogues exhibit a

small percentage of thiamine activity in the curative test of pigeon beri-beri. It is, however, questionable whether such small effects should be considered, since ABDERHALDEN and ABDERHALDEN (1939) believed themselves able to show that rat and pigeon tissues can sometimes synthesize traces of thiamine from the pyrimidine and the thiazole parts of the molecule (with about 1 % yield).

Summarizing the available data on animal experiments with substituted thiamines, SEXTON (1953) came to almost the same conclusions concerning the specificity of the molecule as the observers working with *Phycomyces*.

The sulfur atom of the thiazole moiety was completely neglected by these early workers. Thus the possible homomeric (Fig. 1*d*) and heteromeric (e.g. Fig. 1*e*, *f* and *g*) disulfides of thiamine, while also analogues, do not occur in any of the above-mentioned technical or reviewing papers. We will deal with these derivatives after giving a brief account of theories of the chemical function of thiamine.

## CHEMICAL ACTIVITY OF THIAMINE IN RELATION TO ITS PHYSIOLOGICAL ROLE

The numerous chemical model systems intended to illustrate the theories of thiamine function can be classified according to which moiety of the molecule was regarded as the bearer of the active centrum. Thus the pyrimidine was chosen by LANGENBECK (1933, 1935) who believed in the formation of a Schiff's base between pyruvate and the 4′-amino group. This was, however, disproved by STERN and MELNICK (1939). WIESNER and VALENTA (1956) suggested also a Schiff's-base formation which tautomerizes with the 7′-methenyle bridge which in turn forms an ylid with the 3-N of the thiazole part, without, however, bringing new evidence for the altered theory.

More important are the schemes based on the peculiar thiazole moiety of thiamine. LIPMANN (1937) postulated a redox mechanism between thiamine and 2-dihydrothiamine produced through hydrosulfite treatment. However, KARRER, GRAF and SCHUKRI (1945) have shown that no dihydrothiamine resulted from this reaction, but only cleavage products. Later KARRER (1947) became interested in the possible role of the free sulfhydryl group of the thiole form of thiamine.

The possibility of sulfhydryl/disulfide function between the thiazole and thiole form of thiamine was first presented by ZIMA and WILLIAMS (1940) and was developed into a full theory of cocarboxylase action by MYRBÄCK and VALLIN (1944), who also considered the opportunity of explaining the Pasteur effect through this redox system (cf. Fig. 1*a*, *b*, *c* and *d*).

A rather different approach was published by MIZUHARA and HANDLER (1954), who discovered a nonenzymatic acetoin-formation reaction with thiamine as a catalyst and suggested an acetyl carbanion intermediate which adheres to the Zwitterion of the 2-C and 3-N of the deprotonated thiazole. This reaction, like that in the first theory of BRESLOW (1956) on an ylid between 7′-methenyl and 3-N, became unlikely after the work of INGRAHAM and WESTHEIMER (1956), who recorded only a small deuterium uptake under these conditions.

On the other hand, the deuterium exchange at the 2 position of the thiazole was unexpectedly high in BRESLOW's extended work (1957*a*) and this led to a revised theory, according to which the site of the attachment of the decarboxylated $\alpha$-keto acids in the thiamine molecule must be the 2-C atom (BRESLOW 1957*b*, 1958; BRESLOW and MCNEILS, 1959). Thus on the basis of model experiments on the reactivity of the particular atoms of different thiazoles, an active centrum was pointed out at a place which was always considered to be unoccupiable by anything but a single hydrogen atom if biological activity were to be preserved. The second BRESLOW model found experimental confirmation recently in the work of KRAMPITZ et al. (1958), who synthesized a presumed intermediate DL-2-$\alpha$-hydroxyethyl-thiamine, the activity of which was equal to that of thiamine in a catalytic model system. It was also reported to be active microbiologically for *L. fermenti* and *L. viridiscens* up to about 80 per cent of the potency of equimolar thiamine. It was pointed out in this connection that the product was a racemic derivate. However, this fact should not interfere with the potency of the product, whose deacetylation must necessarily proceed very easily in the cells—if it is a true intermediate—giving rise to full thiamine activity.

Because of this slight contradiction in the interpretations, we tested DL-2-$\alpha$-hydroxyethyl-thiamine (kindly supplied by Professor L. O. KRAMPITZ) in our tube test without cysteine by aseptical addition to the medium (IV). As seen in Fig. 2, the postulated intermediate

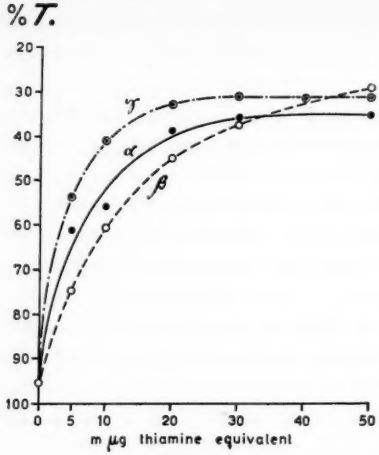


FIG. 2.

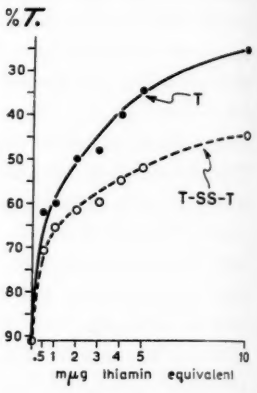


FIG. 3.

FIG. 2. Growth of *Lactobacillus fermenti* 36 with thiamine (α), equimolar amounts of D,L-2-α-hydroxyethyl thiamine of KRAMPITZ et al. (1958) (β) and double equivalent amounts of the same (γ) in the tube test (I, IV) on a cysteine-free basal medium.

FIG. 3. Thiamine disulfide (T-SS-T) supports very scarce growth of the yeast *Klockera brevis* when compared with thiamine (T) in the method of HOFF-JØRGENSEN and HANSEN (1955) but seeding with very fainth inoculum in order to avoid the activation of the disulfide through reduction by the cells.

Readings of triplicates were made in both instances turbidimetrically at 5950 Å and mean values of the percentage of light transmittance (% T.) were plotted against increasing concentrations of the factors in question.

gave curves of different slopes when compared with thiamine. This was the case both at equimolar concentrations and at double concentrations. Thus our results confirm the findings of KRAMPITZ et al. (1958), at least in the respect that a 2-substituted thiamine was found to be fully utilizable by *L. fermenti* under conditions where mainly the monomeric forms of thiamine can promote growth. This fact is rather important if we recall the former opinion that the 2-position of thiamine can be occupied only by a single hydrogen atom (e.g. BERGEL and TODD, 1937).

## DISULFIDE DERIVATIVES OF THIAMINE IN BIOLOGICAL SYSTEMS

The afore-mentioned theory of the –SH/–SS– function of thiamine (or cocarboxylase) presented in detail by ZIMA et al. (1953) could not

stand enzymological trial, as was pointed out in our earlier work (III, IV). KARRER and VISCONTINI (1946), ENGELHARDT and KANOPKAITE (1957) and HOLT et al. (1957), as well as MIZUHARA and HANDLER (1954), found independently that the homomeric disulfides of cocarboxylase or thiamine are completely inert in the unoxidative and oxidative decarboxylation systems as in the nonenzymatic acetoin-formation catalytic system.

Contrary to the above findings, all the biological data pointed in an exactly opposite direction. Thus ZIMA et al. (1953) presented a large amount of evidence on the superiority of the disulfide form in animal and microbiological experiments. As recently as 1956 ZIMA and HOVOTY issued a paper in which the pharmacological superiority of thiamine disulfide over thiamine was claimed because of its lower toxicity, its analgetic effect and its antiphlogistic action in UV burns of the skin.

Several reports have appeared recently on the natural occurrence of the disulfide forms of thiamine and its derivatives. Thus MARTEN (1955) and TAMAKI and NOSE (1955) found disulfides and conversion to disulfides in blood, tissues and organs. SUOMALAINEN, RIHTNIEMI AND OURA (1959) analyzed baker's yeast fluorometrically and came to similar conclusions as MYRBÄCK, VALLIN and MAGNELL (1945), identifying a significant portion of yeast thiamine as cocarboxylase disulfide.

Our earlier report (III) and detailed investigation on *L. fermenti* (IV) gave the first actual biological evidence which is in agreement with the above enzymatic findings. While the results were thoroughly discussed at the time (IV), we can state now that the *per se* inactivity of the homomeric and most of the heteromeric disulfides of thiamine (IV, V, VII) is not restricted to a single bacterial strain. Other heterofermentative Lactobacilli were also found to be unable to utilize the disulfide forms of thiamine unless reduced (VII).

It is maybe still more significant from physiological and nutritional viewpoints that thiamine disulfide was incapable of curing the thiamine deficiency symptoms of the rat at concentrations where thiamine or activated disulfide was of full value (VIII) and that "convection" (nutritional features overshadowed by intestinal symbiosis) does not interfere with this demonstration.

Finally a yeast strain recommended for thiamine assay was chosen for a similar experiment. *Kloeckera brevis* was tested with thiamine

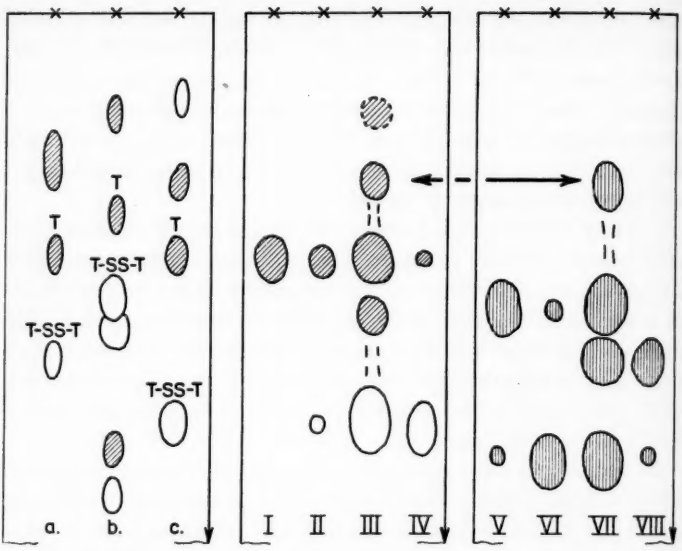


FIG. 4. FIG. 5. FIG. 6.

FIG. 4. Selective bioautographic test of the thiamine-cysteine mixed disulfide complex (MATSUKAWA and YURUGI, 1953) in a, *tert*-butanol : water : methylethylketone (2 : 2 : 1); b, *n*-butanol : ethanol : acetic acid : water (4 : 1 : 1 : 10); and c, *iso*-butanol : pyridine : water (3 : 2 : 5) with *Lactobacillus fermenti* 36 (II, VI, VII). Hatched zones indicate activity both in presence and in absence of cysteine in the basal medium, while unhatched zones are visible only in presence of cysteine, indicating the necessity of activation prior to utilization by the cells. Zones of thiamine (T) and thiamine disulfide (T-SS-T) are marked for comparison.

FIG. 5 and 6. Comparative bioautography of the thiamine–lipoic acid mixed disulfide complex separated in an *iso*-butanol : pyridine : water (3 : 2 : 5) system and developed with *L. fermenti* (Fig. 5) or with *Corynebacterium bovis* (STOKSTAD et. al., 1956) (Fig. 6). Hatching of zones in Fig. 5 should be interpreted as in Fig. 4. The horizontal arrow points to the spots of thiamine lipoate disulfide.

Explanation of the columns: *I*, thiamine; *II*, thiamine at pH 8.9; *III*, thiamine disulfide incubated with dimercaptolipoate (according to VI); *IV*, thiamine disulfide; *V*, dimercapto lipoate; *VI*, lipoic acid disulfide; *VII*, the same as in the third column; *VIII*, lipoic acid sulfoxide. (Paper Whatman Nr. 1).

and thiamine disulfide according to the method of HOFF-JØRGENSEN and HANSEN (1955), except that a rather diluted inoculum was used. As seen in Fig. 3, the scarce availability of the disulfide form for the yeast cells could be clearly demonstrated despite the strong reducing ability of the yeast cells.

We have also shown that a number of mixed disulfides of thiamine

of the allithiamine type (Fig. 1*f*) were similarly inactive for the Lactobacilli (V, VII). More intriguing is, however, the fact that one of the mixed disulfides, viz. the thiamine–pantetheine complex (SAHASHI et al., 1954) (Fig. 1*g*), was found to be active for the bacteria also in the absence of cysteine (VI, VII).

The validity of the BRESLOW theory which places the decarboxylated pyrurate as active acetaldehyde on the 2-carbon atom does not exclude the possibility that the thiamine molecule has a second function through its –SH ending. Thus YATCO-MANZO et al. (1959) found that the pH optimum (8.9) for the catalytic formation of acetoin by thiamine from pyruvate was very close to the average of $pK_2$ and $pK_3$ of thiamine, which implies that thiamine participates in this reaction as the pseudobase (Fig. 1*b*) or the thiole (Fig. 1*c*) but evidently not in its amino form (Fig. 1*a*).

For this reason first the thiamine–cystine (thiamine disulfide–cysteine) reaction of MATSUKAWA and YURUGI (1953) was now followed bioautographically according to the methods detailed in earlier papers (VI, VII, VIII). Thiamine disulfide reacted with cysteine according to the Japanese authors and the reaction mixture was separated on paper chromatograms, as seen in Fig. 4. Besides thiamine one or probably two additional active spots appeared on the agar plates which did not need the presence of additional cysteine in the medium. There are strong indications that we are dealing with a thiamine–cysteine mixed disulfide which will be directly utilized by the cells.

Reduced glutathione was considered to be a reducing agent surpassing cysteine in its capacity to activate thiamine disulfide in the thiochrome test (ZIMA, RITSERT and MOLL, 1941). For this reason SAHASHI et al. (1954) investigated the possibility of mixed disulfide formation between thiamine and this tripeptide and reported the complex disulfide formation of it with thiamine, detected chromatographically with differentiating color reactions. We repeated this work and searched for a microbiologically active disulfide between thiamine and glutathione, however, without result despite numerous attempts.

On the other hand, incubation of thiamine disulfide (prior to aseptical addition to the basal medium) in conditions favoring the thiazole conversion to thiole with equimolar amounts of sulfhydryl compounds resulted in activation of the disulfide but in increasing

order for cysteine, pantetheine, glutathione-SH and lipoic acid dimercaptide. The activation of thiamine disulfide by the dimercaptolipoic acid (1.2-dithiolane-3-valeric acid) was investigated in a manner similar to that used for the work on the thiamine–pantetheine complex. Dimercaptolipoate was prepared from a commercial sample of DL-α-lipoic acid (Nutritional Biochemicals Co., Cleveland, Ohio) according to GUNSALUS, COLOWICK and KAPLAN (1955). For studying the activation effect of dimercaptolipoate on thiamine disulfide, equimolar amounts of the lipoate, calculated on a thiole basis, was allowed to react with the disulfide form of thiamine in a 1/15 M phosphate-buffered solution of pH 7.5 at 37 C for 30 minutes. The activity of this product was tested on a fluid medium in the absence of cysteine. The product was then chromatographed, according to the details in Figs. 5 and 6. Bioautographic development was performed in agreement with former descriptions (II, VI, VII) on media with and without cysteine. Under the circumstances thiamine disulfide is not available to the cells in the usual concentration range, when cysteine is omitted from the medium (III, IV). For the detection of lipoic acid another bioautographic technique was used with *Corynebacterium bovis* 187, (kindly supplied by Dr. E. L. R. STOKSTAD of the Lederle Division, American Cynamide Company, Pearl River, N.Y). The method was based on the agar cup plate test with this organism, according to STOKSTAD et al. (1956), except that the original medium was supplemented with 0.1 % Tween 80.

Comparison of Fig. 5 and Fig. 6 will reveal the fact that the reduced form of lipoic acid formed a conjugate with thiamine, which has a different Rf value than any of the other forms of thiamine and lipoic acid applied. This was confirmed in two different solvent systems. The new product was equally active for both *L. fermenti* and *C. bovis* in the same concentration range as the parent compounds. Under the circumstances thiamine and its derivatives other than thiamine lipoate were completely inactive for *C. bovis*, whilst lipoic acid and its derivatives showed no activity for *L. fermenti* with the exception of the new complex. The new spot showed no fluorescence prior to treatment with cysteine in the thiochrome test, a reaction which is known to fail with the disulfide forms of thiamine. Cysteine treatment of the product of thiamine and lipoic acid will liberate the respective parent compounds.

For the above reasons the new microbiologically active compound

must be considered as a mixed disulfide between thiamine and lipoic acid, and may resemble the suggested formula of Fig. 1*h*. Treatment of thiamine with the disulfide form of lipoic acid will lead to similar results, on the analogy of the reactions between thiamine and cysteine described by MATSUKAWA and YURUGI (1953) and of the interaction between thiamine and pantetheine (VI).

It has not been decided at present which of the –SH groupings of the dihydrolipoate will combine with the –S– of thiamine thiole. However, it can be mentioned in this connection that the –S– atoms of both the 6 and the 8 positions of lipoic acid are able to react with the acyl groups as discovered by GUNSALUS and SMITH (1957). Nevertheless, only the 6-acyl derivative seems to exert metabolic activity in oxidative decarboxylation processes. Thus in a potential intermediate the 8 position should be considered to be available for linkages other than that engaged in acyl transfer, as e.g. for disulfide complex formation with thiamine or cocarboxylase.

Eluting the spot of the probable thiamine lipoate complex and rechromatographing in the same manner resulted in several spots active for *L. fermenti*. One of these was thiamine and the other thiamine disulfide, as shown with their Rf and by inactivity in the absence of cysteine in the second case. The nature of the other factors remained to be clarified. However, the existence of two –SH groups in the lipoic acid molecule will probably explain this multitude of factors observed. The bearing of the present findings on the mechanism of the oxidative decarboxylation processes must be considered in the light of the fact that lipoic acid functions as a catalytic tool receiving the decarboxylated $\alpha$-keto acid from the active cocarboxylase in order to transfer it to coenzyme-A (GUNSALUS and SMITH, 1957).

Isolations of the product described and the testing of its activity as a potential intermediate in enzymatic reactions will be needed before further conclusions can be drawn. On the other hand, the fact should be pointed out that in their early work REED et al. (1951) found lipoic acid to be present in several forms in natural extracts active toward *Streptococcus faecalis*. For some of these naturally occurring metabolites a structure was proposed in which the 4'-amino group of the pyrimidine moiety of thiamine was supposed to be linked to the carboxy ending of lipoic acid (lipothiamine, lipothiamide pyrophosphate) (REED and DEBUSK, 1952). This hypothetical struc-

ture, however, could not be confirmed (REED, 1957). The type of mixed disulfide linkage between thiamine and lipoic acid might explain some of the results with natural extracts containing a multitude of lipoic acid conjugates, as well as the continuity of reaction sequence in oxydative decarboxylation of α-keto acids, where the participation of a thiamine lipoate type of intermediate could easily be imagined.

The possibility that thiamine may form microbiologically active mixed disulfide complexes with cysteine, pantetheine (VI) and lipoic acid opens some new aspects for the consideration of thiamine action in the cells. It is rather important to realize that such active complex formation might also occur between a potential intermediate and the above intermediary metabolites of thiole type. Incidentally we already have preliminary results at hand which show that the biologically active mixed disulfides of the postulated "active acetaldehyde" (D,L-2-α-hydroxyethyl thiamine, KRAMPITZ et al. 1958) behave distinctly differently from those of thiamine in respect of chromatographic motility but are directly comparable with the latter in respect of microbiological activity.

These facts should also be considered as a warning against too rapid conclusions about the discovery and identification of the "natural intermediate" (active acetaldehyde) in tissues and organs. Such a preliminary attempt was recently communicated (CARLSON and BROWN, 1960) and the identification was based on Rf values in six different solvent systems. In this work both of thiamine and D,L-2-α-hydroxyethyl thiamine gave one characteristic spot when detected bioautographically. In our work neither of these substances gave less than two or three spots despite purification. Also the introduction of active mixed disulfides increases the number of possible spots, the comparison of which with those in natural products can hardly be considered as a sufficient measure for a final identification. For the illustration of the possible large number of thiamine components in a natural extract, the work of IACONO and JOHNSON (1957) should be considered; they found at least eight microbiologically active unidentified thiamine derivatives in the rat urine.

## SUMMARY

A critical survey (based on earlier publications I to VIII) was given of current work dealing with the question of utilization of different homomeric disulfide forms of thiamine and its derivatives, as well as that of some mixed disulfides of thiamine by bacterial cells and by the rat.

At variance with the present belief on the biological behavior of the disulfides of thiamine we found that activation through reduction (e.g. by cysteine, ascorbic acid, etc.) is necessary to render the disulfides available for these organisms.

Similar results are given here for the yeast *Kloeckera brevis*.

The biological inactivity of most of the disulfide derivatives points to a general rule which is in agreement with the enzymatic model experiments hitherto published. However, some peculiar exceptions to this rule were found. Thus besides the thiamine-pantetheine reaction already described (VI) the active mixed disulfide formation between cysteine and thiamine and between lipoic acid and thiamine (thiamine lipoate) was detected. These complex compounds can also be utilized by the bacterial cells in absence of reducing agents.

Thiamine lipoate disulfides might explain the original observation of the presence of a large number of lipoic acid conjugates in natural extracts, instead of the postulated lipothiamide, the existence of which remained unproved.

The occurrence of active mixed disulfides of thiamine implies the possibility of an additional biochemical mechanism in the cells which need not necessarily be in disagreement with the main function of thiamine suggested by BRESLOW (1958). This suggestion found further support when D,L-2-α-hydroxyethyl thiamine was tested with *Lactobacillus fermenti* and results were obtained similar to those of KRAMPITZ et al. (1958) also in our chemically defined medium without cysteine.

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